Am J Transl Res 2016;8(5):2059-2069
www.ajtr.org /ISSN:1943-8141/AJTR0011883

Original Article

Rat adipose-derived stem cells express low level of α-Gal and are dependent on CD59 for protection from human xenoantibody and complement-mediated lysis

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Received June 24, 2015; Accept July 31, 2015; Epub May 15, 2016; Published May 30, 2016

Abstract: Since increasing evidence has indicated that adipose-derived stem cells (ASCs) can function across the species barrier, the use of xenogeneic ASCs may be a practical alternative to the autotransplantation and allotransplantation. Before animal ASCs can be used clinically, evidence needs to be provided to indicate whether they will survive in a human host. In the present study, we investigated whether rat ASCs (rASCs) could resist human xenoantibody and complement-mediated lysis as well as investigated the possible mechanisms involved. We found that rASCs could significantly resist human natural antibody and complement-mediated cytotoxicity when incubated with 20% or 50% normal human serum (NHS) *in vitro*, as compared with rat lymphocytes (rLCs). Mechanistically, rASCs expressed lower level of xenoantigen Galα1-3Galβ1-4GlcNAc (α-Gal), which was correlated with decreased binding of human xenoreactive IgG and IgM and reduced deposition of complement C3c and C4c. More interestingly, rASCs had minimal deposition of human membrane attack complex (C5b-9). When the expression of CD55 and CD59 was analyzed by flow cytometry, we found that rASCs expressed very weak CD55 but expressed much higher level of CD59 than rLCs. Moreover, the knockdown of CD59 expression by siRNA largely reversed the resistance of rASCs to the human serum-mediated lysis. Taken together, these data have demonstrated for the first time that rat ASCs are capable to protect themselves from human xenoantibody and complement-mediated lysis, which is dependent on CD59 and is correlated with low expression of α-Gal. Xenogenic ASCs may have the potential to treat patients in the future.

Keywords: Adipose-derived stem cell, xenotransplantation, antibody mediated complement-dependent cytotoxicity, CD59

Introduction

Ever since Zuk et al. first discovered the multipotent stem cells in adipose tissue [1], interest is growing in the use of adipose-derived stem cells (ASCs) for clinical applications. Compared with bone-marrow-derived mesenchymal stem cells (MSCs), ASCs possess the same capacity in yielding of adherent stromal cells, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction efficiency [2]. Meanwhile, with advantage in harvest convenience [3] and high *in vitro* proliferation rate [4], adipose-derived stem cell currently represents one of the most ideal material in tissue engineering and cell therapy.

In addition to the capability of multilineage differentiation and promoting neovascularization, ASCs can modulate immune system [5]. It has been reported that ASCs can inhibit lymphocyte activation and proliferation [5], induce $CD4^+CD25^+FoxP3^+$ regulatory T cells [6], and down-regulate the costimulatory molecules in dendritic cells [7]. Additionally, ASCs has been found to produce some immunoregulatory cytokines and factors, such as interleukin-10 [8], indoleamine 2,3-dioxygenase (IDO) [8] and Prostaglandin E2 [9]. The Immunomodulatory properties of ASCs may contribute to its utility in transplantation, autoimmune diseases and inflammatory disorders. Recent studies have demonstrated that autogeneic or allogeneic

ASCs transplantation is a promising therapy for various pathologic conditions, such as autoimmune diseases including rheumatoid arthritis [10] and systemic lupus erythematosus [11], ischemia disorders including ischemic limb diseases [12] and myocardial ischemic diseases [13], and islet transplantation [14].

Since increasing evidence has indicated that ASCs can function across the species barrier, the use of xenogeneic ASCs may be a practical alternative to the autotransplantation and allotransplantation. Xenotranplantation with human ASCs has been found to improve neurological functions in a cerebral ischemic rat model [15], maintain glucose level in type 1 diabetes mouse [16], modulate callus induction in bone fracture tissue [17] and ameliorate GVHD in mouse [18]. However, whether the clinical application of animal ASCs is effective and available is not clear. Before animal ASCs can be used clinically, evidence needs to be provided to indicate whether they will survive in a human host. In human-to-rodent ASCs xenotransplantation, the absence of anti-human preformed xenoreactive antibodies in rodents may facilitate the survival of human ASCs. In contrast, since most rodent cells express xenoantigen Galα1-3Galβ1-4GlcNAc (α-Gal) while human sera contain abundant preformed anti-α-Gal antibodies, rodent ASCs may be rapidly destroyed in human host due to complement activation after xenotransplantation. In the present study, we investigated whether rat ASCs could resist human xenoreactive antibodies and complement-mediated lysis as well as investigated the possible mechanisms involved.

Materials and methods

Animals

Four-week-old male Sprague-Dawley (SD) rats (80-120 g) were used as ASC donors. All animals were obtained from Tongji Medical College, Huazhong University of Science and Technology (Wuhan, Hubei, China) and maintained under specific pathogen-free conditions. All of the experiments were performed under the guidelines of Tongji animal use regulations and approved by the institutional animal care and use committee (IACUC) at the Tongji Medical College, Huazhong University of Science and Technology. All surgery was performed under sodium pentobarbital anesthesia, and efforts were made to minimize suffering.

Cell isolation and culture

Rat ASCs (rASCs) were prepared according to published methods [19-21] with modifications. In brief, rat inguinal subcutaneous adipose tissue was excised after sacrifice of the rats. The adipose tissue was minced and then digested in 0.1% collagenase I solution (Sigma, St. Louis. Mo.) at 37°C for 40 min. The suspension was filtered through a nylon screen with a pore size of 74 μm to remove tissue debris and then centrifuged at 720 g for 10 min. The cell pellet was resuspended in a complete cultural medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and penicillin/streptomycin (Hyclone, China). After incubated at 37°C for 24 h in a humidified atmosphere containing 5% CO_2, cells were washed extensively to remove non-adherent cells. rASCs from passages 2 to 5 were used in the subsequent experiments.

As control cells, rat lymphocytes (rLCs) were isolated from buffy-coat preparations of blood using density gradient centrifugation with rat lymphocyte isolation medium (TBD, China).

Identification and differentiation

rASCs (2×10^5 cells/tube) were labeled for 30 min at 4°C in FACS (Fluorescence Activated Cell Sorter) buffer (PBS contained 2% FBS and 0.02% azide) with manufacturer-recommended concentrations of fluorescence-labeled mouse-anti-rat antibodies including fluorescein isothiocyanate (FITC)-conjugated CD44, phycoerythrin (PE)-conjugated CD45, PE-conjugated CD90 (1:100, BD Pharmingen, USA) and PE-conjugated MHC-II (0.3:100, eBiosciences, USA). Cells incubated with FACS buffer alone were used as negative controls. The cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences).

To perform differentiation experiments, cells were replated at the density of 2×10^4 cells/cm^2 for adipogenic differentiation and 5×10^3 cells/cm^2 for osteogenic differentiation once cells were approximately 80-90% confluent. rASCs underwent a 3-week induction of differentiation in either adipogenic induction medium or

osteogenic induction medium. The adipogenic induction medium used in this study was DMEM supplemented with 10% FBS, 0.5 mM isobutylmethylxanthine (IBXM), 1 μM dexamethasone, 10 μM insulin, and 200 μM indomethacin. The osteogenic induction medium was DMEM supplemented with 10% FBS, 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate, and 10 mM beta-glycerophosphate. Cells after differentiation were characterized by oil red O staining or alizarin red staining respectively.

Blood and serum preparations

Fresh non-anticoagulated human blood was obtained from six healthy volunteers with blood type AB, who had given informed consent in accordance with the Helsinki Protocol and received no medication for at least 10 days. Complement-active normal human AB serum (NHS) was collected, pooled, and then stored at -80°C, to maintain the complement activity. As a negative control, pooled NHS was decomplemented by heat at 56°C for 30 min, referred as heat inactivated normal human serum (HINHS).

Antibody-mediated complement-dependent cytotoxicity (CDC) assay

The CDC assay was performed by flow cytometry to address whether rASCs could resist the human xenoreactive antibodies and complement-mediated lysis. Briefly, equal amount of rASCs or rLCs (2×10^5) were incubated with 20% or 50% pooled NHS at 37°C for 2 h. After incubation, the cells were washed twice and then resuspended in 200 μL FACS buffer. Seven microliters of propidium iodide (PI, final concentration 12.5 μg/mL, BD Pharmingen, USA) was then added into each tube. After further incubation in the dark for 12 min at room temperature, the cells were resuspended in a total volume of 500 μL, and then analyzed by flow cytometry (FACSCalibur, BD Biosciences). The percentage of PI-positive cells of the total cells was compared. Cells incubated with 20% HINHS acted as negative controls. Cell number of each tube was counted microscopically before the analysis by flow cytometry.

Detection of C3c, C4c and C5b-9 deposited on cells

The deposition of C3c, C4c, and C5b-9 (membrane attack complex, MAC) on rASCs or rLCs after incubation with 20% NHS was detected by flow cytometry as described previously [22]. Briefly, rASCs or rLCs (2×10^5/tube) were incubated with either 20% NHS or 20% HINHS at 37°C for 15 min. To detect C3c and C4c deposition, cells were incubated with FITC-conjugated mouse anti-human C3c or C4c antibody (1:100, Zhongshan Biotechnology, Beijing, China) at 4°C for 30 min. To detect C5b-9 deposition, cell were incubated with mouse anti-human C5b-9 primary antibody (1:100, DAKO Corporation, CA, USA) at 4°C for 30 min, followed by the FITC-conjugated rabbit anti-mouse secondary antibody (1:100, Zhongshan Biotechnology, Beijing, China) at 4°C for 30 min. Cells incubated with 20% HINHS served as negative controls. The stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). The geometric mean fluorescence intensity (Gmean) of each kind of cells was compared.

Binding of xenoreactive antibodies present in human serum

To detect the binding of preformed xenoreactive IgG and IgM to rASCs or rLCs, 2×10^5 non-fixed single cells were incubated with HINHS (1:40 dilutions) at 4°C for 30 min, washed, and then incubated with FITC-conjugated goat-anti-human IgG or IgM (1:100, Boster, Wuhan, China). rASCs or rLCs incubated with secondary antibody alone served as negative controls. The stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). The Gmean was used to evaluate the degree of xenoantibody binding to each kind of cells.

Detection of α-Gal by flow cytometry

The α-Gal expression on the cell surface of rASCs and rLCs was analyzed by flow cytometry. Appropriate rASCs or rLCs (2×10^5) were incubated with FITC-conjugated BS-IB4 (5 μg/ml, Sigma-Aldrich Corp., USA) for 30 min at 4°C. The stained cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). The Gmean of each kind of cells was compared.

Detection of CD55 and CD59 expression on cells

The difference of CD55 and CD59 expression between rASCs and rLCs was analyzed by flow cytometry. To detect CD55 expression, 2×10^5 of rASCs or rLCs were incubated with rabbit-

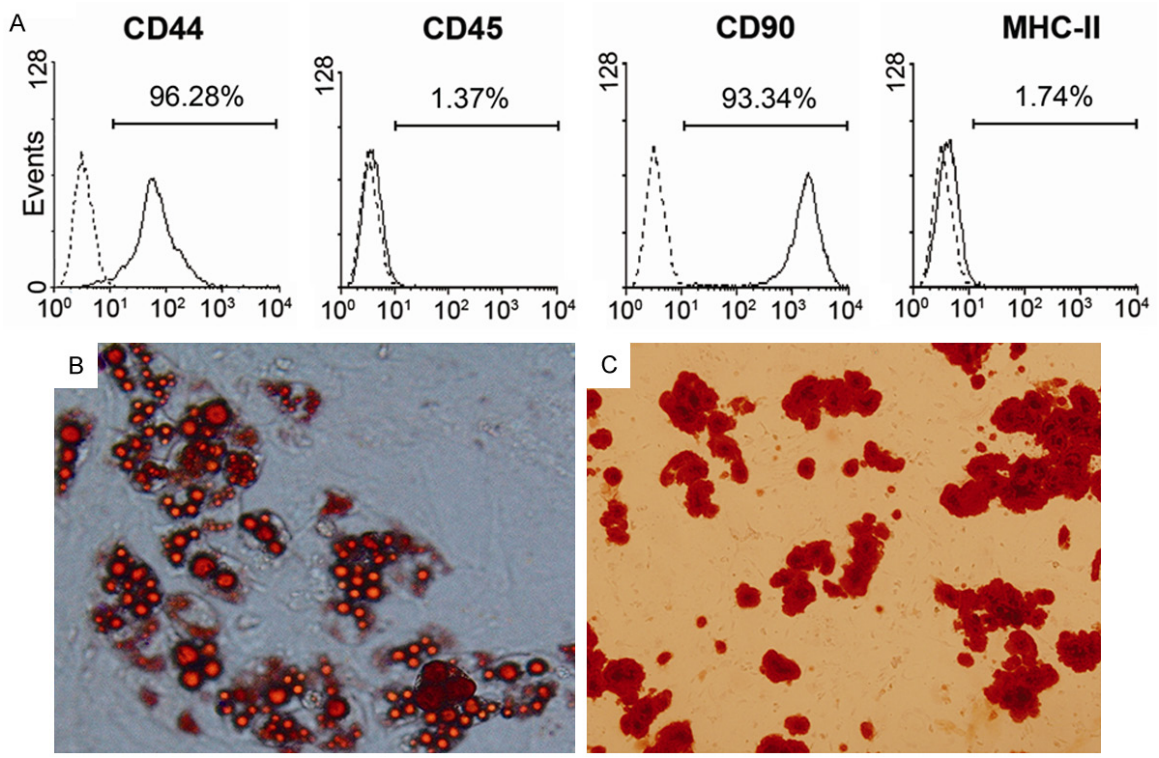

Figure 1. Characterization of the cultured rASCs. A. Cell surface makers (CD44, CD45, CD90, and MHC-II) were analyzed by flow cytometry. B. Adipogenic differentiation was indicated by the accumulation of neutral lipid vacuoles that stained with oil red O. C. Osteogenic differentiation was indicated by calcium deposition stained with alizarin red.

anti-rat CD55 primary antibody (Santa Cruz, Dallas, USA) at 37°C for 30 min, followed by FITC-conjugated goat-anti-rabbit secondary antibody (1:100, Zhongshan Biotechnology, Beijing, China) at 37°C for 30 min. Cells incubated with secondary antibody alone served as negative controls. To detect CD59 expression, 2×10^5 of rASCs or rLCs were incubated with peridinin chlorophyll protein (PerCP)-conjugated mouse-anti-rat CD59 (2.5:100, eBiosciences, USA) at 37°C for 30min. Cells incubated with FACS buffer alone acted as negative controls. After staining, the cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences). The Gmean of each kind of cells was compared.

siRNA transfection

The target mRNA sequences of the rat CD55 and CD59 genes were selected. The oligonucleotides contained sense and antisense of the target sequences and restriction enzyme sites at the end of the strands. The sequences were: 1) targeting the CD55 gene: 5'-GCUUCAUUCUGGUUGGAAA dTdT-3' (sense), 3'-dTdT CGAAGUAAGACCAACCUUU-5' (anti-sense); and 2) targeting the CD59 gene: 5'-CCUGGAUGCUUGUCUUGUU dTdT-3' (sense), 3'-dTdT GGACCUACGAACAGAACAA-5' (anti-sense). The oligonucleotides and control siRNAs were synthesized by Ribobio, Guangzhou, China. A mixture of CD55-siRNA or CD59-siRNA and lipofectamine iRNAMax (Invitrogen, USA) was prepared before rASCs trypsinization. The cells were transfected with the siRNA mixture for 72 h. Gene silencing was evaluated by Quantitative real-time PCR (QPCR) at 24 h and flow cytometry at 72 h. Specific siRNA-treated rASCs were used for CDC experiments as described above.

Detection of CD55 and CD59 gene silencing by QPCR

Total RNA was extracted from rASCs using Trizol reagent (Invitrogen, USA) and mRNA was reverse-transcriped with the cDNA synthesis kit (Promega, WI). cDNA was synthesized from 1 μg of total RNA. The reaction protocol included a 10-min incubation at 95°C, then 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 20 sec, followed by a final elongation at 72°C for 5 min. The following paired primers were

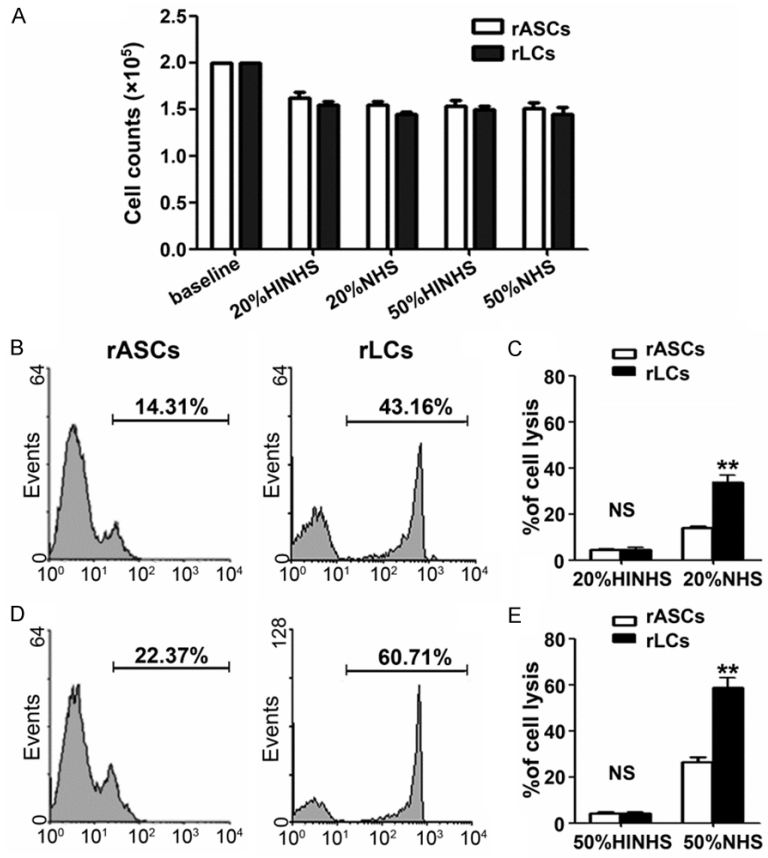

Figure 2. rASCs resist the human xenoreactive antibody and complement-mediated lysis (CDC). Cell death was analyzed by flow cytometry after the incubation of rASCs or rLCs with 20% or 50% NHS. Cells incubated with 20% or 50% HINHS acted as negative controls. (A) Cell count of each tube before and after the CDC procedure; (B, D) After the incubation with 20% (B) or 50% (D) NHS, cells were analyzed by FACS. Results showed are representative of three independent experiments; (C, E) The CDC levels of each group after the incubation with 20% (C) or 50%, E NHS are shown as bar graphs. Data shown are means ± SEM (**P<0.01, n = 3 per group).

used to detect β-actin: 5'-CGTTGACATCCGTAAAGACCTC-3' (forward) and 5'-TAGGAGCCAGGGCAGTAATCT-3' (reverse); CD55: 5'-AAGGCTTCATTCTGGTTGGA-3' (forward) and 5'-TCTGGTGGGGTCTTAGTTGG-3' (reverse); CD59: 5'-GAGGTTGCAGATTTGGTGGA-3' (forward) and 5'-GCATGAAGAAACCGGGTCTAA-3' (reverse). Quantitative real-time PCR reactions were performed in a Step one plus real-time PCR system (Applied Biosystems, CA) using SYBR green master mix (TAKARA, Japan). β-actin was used for sample standardization. Fold change was calculated using the ΔCt value.

Statistical analysis

The data are indicated as the means ± S.E.M. All of the statistical analyses were performed using GraphPad Prism V5.0 (GraphPad Software, USA). The significance of differences between selected groups was evaluated using Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

Characterization of rASCs

The cultured rASCs exhibited the expected spindle-shaped, fibroblast-like morphology and expressed the phenotypic markers shown in **Figure 1A**. In consistence with previous report [23], our results indicate that the rASCs were positive for cell adhesion molecules CD44 and the stromal marker CD90, while they did not express MHC class II markers and the CD45 hematopoietic lineage marker. When cultured in lineage-specific differentiation culture medium, rASCs within 2-5 passages could undergo adipogenic (**Figure 1B**) and osteogenic (**Figure 1C**) differentiation.

rASCs resist the human xenoreactive antibody and complement-mediated lysis

To investigate whether rASCs could resist the human xenoreactive antibody and complement-mediated cell lysis, the CDC assay was performed by flow cytometry with 20% or 50% NHS as source of natural antibodies and complement. The cell number of each tube had a decrease of 20% to 25% after the CDC procedure, but no significant difference was shown between the incubation with HINHS and NHS, indicating that minimal cells were completely lyzed by the complement after the incubation at 37°C for 2 h (**Figure 2A**). After incubation with either 20% or 50% NHS, the CDC level of the rASCs was significantly lower than that of the rLCs (*P<0.01*) (**Figure 2B-E**). When treated with 20% or 50% HINHS, both cells displayed only background cytotoxic activity (**Figure 2C** and **2E**).

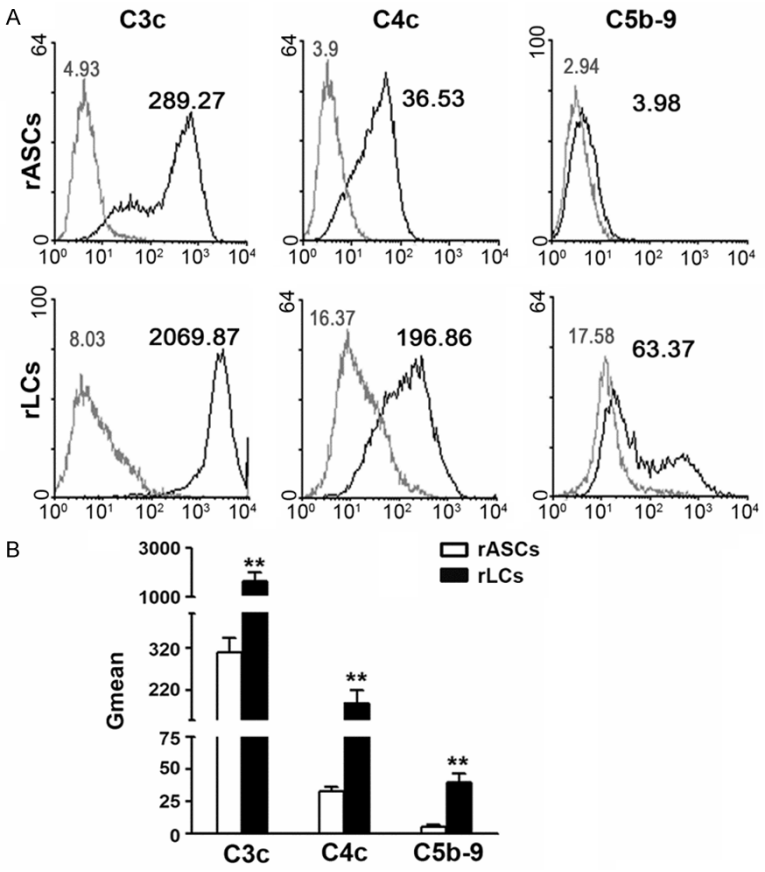

Figure 3. rASCs inhibit the formation of membrane attack complex. Deposition of complement fragments C3c, C4c, and C5b-9 (MAC) on rASCs or rLCs after incubation with 20% NHS was detected by flow cytometry and was evaluated by the geometric mean fluorescence intensity (Gmean). Negative controls were cells incubated with 20% HINHS. A. FACS results shown are representative of three independent experiments; B. The degrees of C3c, C4c, and C5b-9 deposition on each kind of cells are shown as bar graphs. Data shown are means ± SEM (***P*<0.01, n = 3 per group).

rASCs are able to inhibit the formation of membrane attack complex

To study the mechanism for rASCs resistance to human xenoantibody and complement-mediated lysis, deposition of C3c, C4c, and C5b-9 (MAC) on rASCs or rLCs after incubation with 20% NHS was detected by flow cytometry. As shown in **Figure 3**, much less C3c, C4c, and C5b-9 deposition were found on rASCs than that on rLCs. More impressively, the deposition of C5b-9 on rASCs was almost absent, suggesting that rASCs are able to inhibit the formation of membrane attack complex, a mechanism that may protect the cells against xenoantibody and complement-mediated humoral injury.

Less binding of human xenoreactive antibodies to rASCs

Since rASCs had much less C3c and C4c deposition than rLCs after incubation with NHS, the capability of rASCs to bind xenoreactive antibodies present in human serum might be much weaker than that of rLCs. After incubation with the same diluted pooled HINHS, the binding of human IgG and IgM on both cells was analyzed by flow cytometry and evaluated by Gmean values, respectively. Although both rASCs and rLCs could be recognized by human xenoreactive IgG and IgM, the binding of human serum IgG and IgM with rASCs was both significantly lower than that with rLCs (IgM: 9.03±1.45 vs. 190.70±55.15, *P<0.05*; IgG: 5.73±1.01 vs. 27.49±3.90, *P<0.01*) (**Figure 4**).

Lower α-Gal expression on rASCs

As rASCs had a lower capability to bind human xenoreactive antibodies than rLCs, it is possible that rASCs express lower levels of the xenoantigen α-Gal as compared with rLCs. Both cells were analyzed by flow cytometry and the Gmean values were used to evaluate the level of α-Gal expression on the cell surface. As shown in **Figure 5**, the α-Gal expression on rASCs (13.97±0.32) was significantly lower than that detected on control rLCs (24.47±3.03) (*P<0.05*).

Higher CD59 expression on rASCs

To address a possible reason for why rASCs are able to prevent MAC formation but rLCs do not, the expression of 2 complement regulatory factors (CD55 and CD59) on both cells were assayed by flow cytometry and evaluated by Gmean values, respectively. The results showed that the expression of CD59 was much

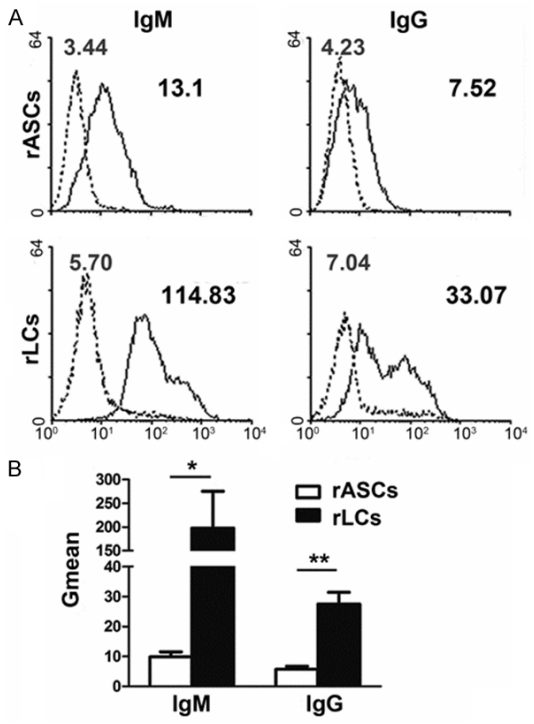

Figure 4. Less binding of human xenoreactive antibodies to rASCs. After incubation with 2.5% HINHS, the binding of human IgG and IgM on rASCs or rLCs was analyzed by flow cytometry, respectively. The Gmean was used to evaluate the degree of xenoantibody binding to each kind of cells. Cells incubated with secondary antibody alone served as negative controls. A. FACS results shown are representative of four independent experiments; B. The degrees of human IgG and IgM binding on each kind of cells are shown as bar graphs. Data shown are means ± SEM (*P<0.05, **P<0.01, n = 4 per group).

higher on rASCs (75.87±3.81) than that of rLCs (7.50±0.96) (*P<0.01*), whereas the expression of CD55 was very weak on both cells (P>0.05) (**Figure 6**).

CD59 plays a critical role in the protection of rASCs from human xenoreactive antibody and complement-mediated lysis

Since much higher CD59 expression was found on rASCs than that of rLCs, CD59 may play an important role in the protection of rASCs from human xenoreactive antibody and complement-mediated lysis. To confirm this hypothesis, specific siRNA targeting either CD59 or CD55 was designed and applied to knockdown the expression of CD59 and CD55, respectively. QPCR revealed that both CD59-siRNA and CD55-siRNA could effectively down-regulate the corresponding mRNA expression in rASCs (**Figure 7A**). Moreover, CD59-siRNA significantly attenuated the protein expression of CD59 on the surface of rASCs (**Figure 7B**). After transfected with either CD59-siRNA or CD55-siRNA for 72 h, rASCs were incubated with 20% NHS for the CDC assay. When compared to untreated rASCs and control siRNA-treated rASCs, the CDC level of CD59-siRNA-treated rASCs significantly elevated to a level similar to that of rLCs (*p<0.05*) (**Figure 7C** and **7D**). In contrast, no increase of cytotoxicity was observed in rASCs transfected with CD55-siRNA (*P>0.05)* (**Figure 7C** and **7D**). Taken together, these results clearly demonstrated that CD59 is responsible for the resistance of rASCs to the human xenoantibody and complement-mediated cytotoxicity.

Discussion

Since ASCs have been demonstrated to be able to function across the species barrier, the use of xenogeneic ASCs may be a practical alternative to the autogeneic and allogeneic ASCs for the purpose of cell therapy. Animal ASC is a promising material in MSC transfusion therapy due to the feasibility of genetic modification and thus has advantage in being used as a vehicle in gene therapy and drug delivery [24]. Before animal ASCs can be used clinically, evidence needs to be provided to indicate whether they are capable to protect themselves from human serum-mediated lysis. In this study, we demonstrated for the first time that rat ASCs could resist human xenoantibody and complement-mediated lysis and identified the possible mechanism of resistance as being related to lower α-Gal expression and higher CD59 expression as compared with control rat lymphocytes.

The α-Gal epitope is abundantly expressed on most cells in non-primate mammals and New World monkeys. In contrast, this epitope is not expressed on cells from Old World monkeys, apes or humans; instead, they produce enormous amounts of anti-Gal antibodies against α-Gal epitope [25]. In the situation of rat-to-human cell xenotransplantation, the preformed anti-Gal antibodies contained in human serum will bind to the α-Gal epitopes expressed on rat cells, resulting in the activation of complement via the classic pathway and subsequently causing the rapid cell death. In this study, the CDC

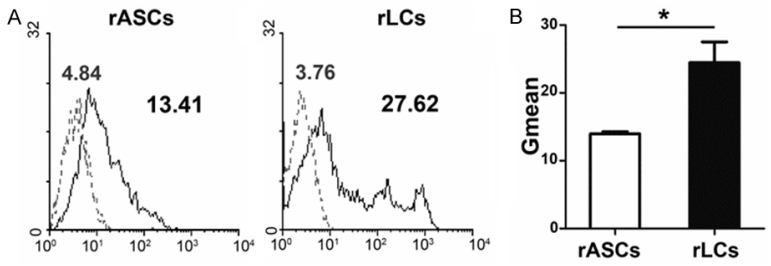

Figure 5. rASCs express lower level of α-Gal. rASCs or rLCs stained with BSI-B4 were analyzed by flow cytometry and the Gmean values were used to evaluate the level of α-Gal expression on the cell surface. Cells without staining were used as controls. A. FACS results shown are representative of three independent experiments. B. The levels of α-Gal expression on each kind of cells are shown as bar graphs. Data shown are means ± SEM (*P<0.05, n = 3 per group).

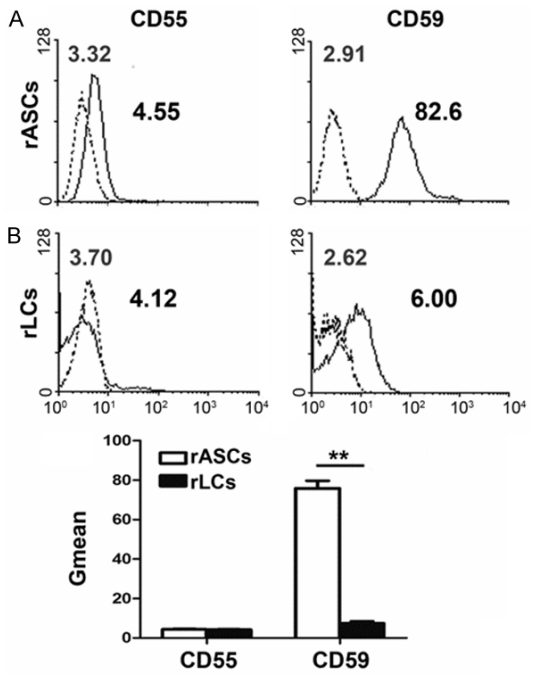

Figure 6. rASCs express higher level of CD59. Flow cytometry was used to detect CD55 and CD59 expression on rASCs or rLCs. Cells without staining were used as controls. The Gmean values were used to evaluate the CD55 and CD59 expression on each kind of cells. A. FACS results shown are representative of three independent experiments. B. The CD55 and CD59 expression on each kind of cells are shown as bar graphs. Data shown are means ± SEM (*P<0.01, n = 3 per group).

assay was performed on rASCs by flow cytometry with 20% or 50% normal human serum as source of xenoantibodies and complement. To our surprise, the CDC level of the rASCs was significantly lower than that of control rLCs, indicating that rASCs can protect themselves against human xenoantibodies and complement-mediated cytotoxicity. Compared with rLCs, rASCs may express less α-Gal, and thus bind less xenoreactive antibodies, which results in attenuated activation of complement. With the analysis by flow cytometry, our results did show that rASCs expressed less α-Gal than that of rLCs, which may contribute to the reduced CDC of rASCs. Additionally, after the incubation with 20% pooled NHS, less binding of human xenoantibodies and less binding of complement C3c abd C4c on rASCs than rLCs was observed, possibly due to reduced α-Gal expression on the rASCs.

Although rASCs were found to express lower intensity of α-Gal and bind less human xenoantibodies than rLCs, the complement cascade was still partially activated via the classical pathway as indicated by deposition of the complement fragments C3c and C4c. However, deposition of MAC (C5b-9) was almost absent on rASCs, whereas it was strongly positive on rLCs. This would suggest that rASCs are able to inhibit MAC formation by some other unknown mechanisms. Complement regulatory proteins can inhibit complement activation, which may contribute to the suppression of MAC formation after the incubation of rASCs with 20% NHS. It was reported that human MSCs lacked expression of MCP (CD46) and DAF (CD55), but were protected from complement lysis via expression of protectin (CD59) [26]. Other studies demonstrated that Human ASCs could also express CD59 [27-29]. Whether rat ASCs could express CD59 and by which offer effective protection across the species barrier is still unknown. In this study, the expression of CD55 and CD59 on rASCs and rLCs were assayed by flow cytometry. We found that the expression of CD59 was much higher on rASCs than that of rLCs, whereas the expression of CD55 was minimal on both cells. These findings suggest that rat CD59 expressed on rASCs may play an important role in the prevention of human MAC formation. To confirm this hypothesis, rat CD59-specific siRNA was used to down-regulate the CD59 expression in rASCs and then the CDC assay was performed by flow cytometry

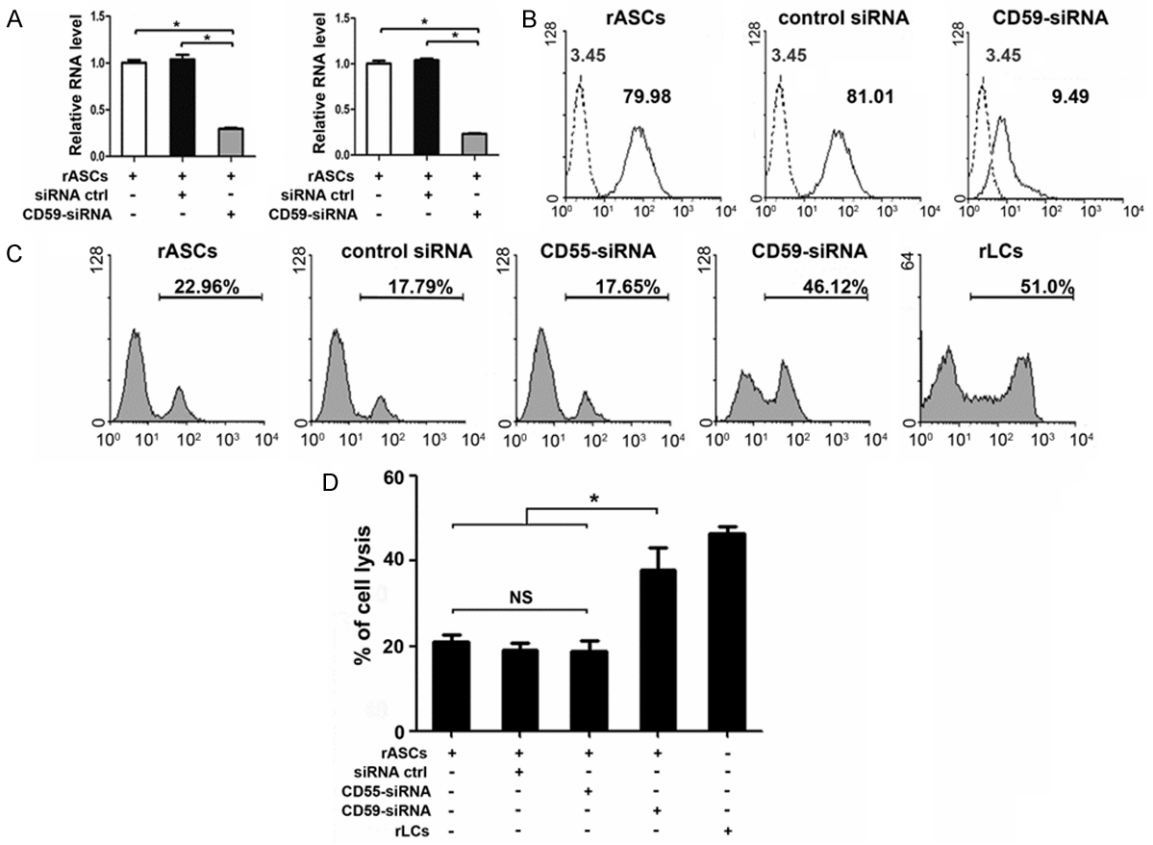

Figure 7. CD59 plays a critical role in protecting rASCs from human xenoantibody and complement-mediated lysis. A. After being transfected with either CD55-siRNA or CD59-siRNA for 24 h, the mRNA expression of CD55 and CD59 in rASCs was accessed respectively by QPCR. Untreated rASCs and control siRNA-treated rASCs were used as controls. Data shown are means ± SEM (**P*<0.05, n = 3 per group). B. After being transfected with CD59-siRNA for 72 h, the protein expression of CD59 on rASCs was measured by flow cytometry. Untreated rASCs and control siRNA-treated rASCs served as controls. The FACS results shown here are representative of three independent experiments. GMean values are shown. C *and* D, After being transfected with CD59-siRNA for 72 h, the rASCs were incubated with 20% NHS to perform the CDC assays. Cell death was analyzed by flow cytometry. Untreated rASCs and rLCs, and control siRNA-treated rASCs served as controls. The FACS results shown here are representative of three independent experiments C. The CDC levels of each cell group are shown as bar graphs D. Data shown are means ± SEM (**P*<0.05, n = 3 per group).

after incubation with 20% NHS. Interestingly, the knockdown of CD59 expression largely reversed the resistance of rASCs to the human xenoantibodies and complement-mediated cytotoxicity, evidenced by the marked elevation of CDC to a level similar to that of rLCs. In contrast, almost no increase of cytotoxicity was observed in rASCs transfected with rat CD55-siRNA. Taken together, these results clearly demonstrated that CD59 plays a critical role in the protection of rASCs from human xenoreactive antibody and complement-mediated lysis. Additionally, our results also indicated that rat CD59 was effective in inhibiting human complement activation at least in vitro.

In the present study, despite a prominent resistance of rASCs to human MAC-mediated lysis through overexpression of rat CD59, rASCs still sustain detectable levels of human xenoantibody binding, as well as complement C3 and C4 fragment deposition on their surface, which may trigger unfavorable immune reactions to the human immune cells present with FC- and/or complement receptors. Thus, it cannot be excluded that the rASCs applied *in vivo* might still be killed via a mechanism of cell-dependent phagocytosis. Whether rat ASCs could resist cell-dependent phagocytosis needs to be further evaluated.

In conclusion, we demonstrated for the first time that rASCs are not susceptible to human natural preformed antibodies and complement-mediated lysis. This self-protective property is correlated with the lower expression of xenoan-

tigen α-Gal and higher production of rat complement regulatory protein CD59. rASCs may have the potential to become a new material in clinical or preclinical application, and more research is needed to better evaluate this therapeutic option.

Acknowledgements

We thank Dr. Deborah McClellan for editorial assistance. This study was supported by the National High-Tech Researching and Developing Program (Program 863) of Ministry of Science and Technology of China (2012AA021010).

Disclosure of conflict of interest

None.

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